Title

Characterization of the maintained vegetative phase deletions from diploid wheat and their effect on VRN2 and FT transcript levels

Journal

Molecular Genetics and Genomics, 283(3)

ISSN

1617-4615

Authors

Distelfeld, A
Dubcovsky, J

Publication Date

2010-03-01

DOI

10.1007/s00438-009-0510-2

Peer reviewed

Mol Genet Genomics (2010) 283:223–232
DOI 10.1007/s00438-009-0510-2

ORIGINAL PAPER

Characterization of the *maintained vegetative phase* deletions from diploid wheat and their effect on *VRN2* and *FT* transcript levels

Assaf Distelfeld · Jorge Dubcovsky

Received: 19 October 2009 / Accepted: 30 December 2009 / Published online: 9 January 2010

Abstract Allelic differences at the *VRN1* (*AP1/CAL/FRU*), *VRN2* (*ZCCT*) and *VRN3* (*FT*) vernalization genes affect flowering time in wheat. The two maintained vegetative phase (*mvp*) mutants from *Triticum monococcum* L., previously reported as carrying a single gene (*VRN1*) deletion, are incapable of flowering. In this study, we show that both *mvp* lines have larger deletions that include the genes *AGLG1*, *CYS*, *PHYC, VRN1* and possibly others. The original *mvp* deletions were generated in lines that lack the *VRN2* gene. Therefore, to study the effect of the *mvp* deletions on the regulation of *VRN2* we generated populations segregating for both genes simultaneously. The two *mvp* deletions co-segregated with the non-flowering phenotype, but surprisingly, the lines homozygous for the *mvp* mutations showed reduced transcript levels of both *VRN2* and *FT* relative to the wild type. The *VRN1* deletion is an unlikely cause of the down-regulation of *VRN2* since *VRN2* transcript levels are higher in the fall, before *VRN1* is expressed, and are down-regulated by *VRN1*. Since both *VRN2* and *FT* are regulated by light and photoperiod, their down-regulation in the *mvp* mutants might be related to the deletion of the *PHYC* photoreceptor. However, alternative hypotheses including combinations of other genes deleted in the *mvp* mutants cannot be ruled out. Until the specific gene(s) responsible for the down-regulation of *VRN2* and *FT* and the non-flowering phenotype are precisely identified, it is premature to use these results to postulate alternative flowering models.

Communicated by A. Schnittger.

A. Distelfeld · J. Dubcovsky (✉)
Department of Plant Sciences, University of California,
One Shields Avenue, Davis, CA 95616, USA
e-mail: jdubcovsky@ucdavis.edu

Keywords Flowering · Wheat · Vernalization · Phytochrome · *VRN1*

Introduction

A precise regulation of flowering initiation is essential for the adaptation of wheat and barley varieties to different environments. Therefore, a better understanding of the regulatory gene network responsible for this developmental transition is required for an efficient manipulation of this trait in changing environments.

The recent discovery of two non-flowering mutants of cultivated diploid wheat (*Triticum monococcum* ssp. *monococcum* L., $2n = 2x = 14$, genome A^mA^m) has provided a new tool to dissect the flowering regulatory network in the temperate cereals. These mutants, designated maintained vegetative phase (*mvp*), were generated by ion-beam radiation and remain indefinitely in the vegetative state independently of environmental stimuli (Shitsukawa et al. 2007).

On the contrary, wild type plants of the same species have the ability to initiate the reproductive phase in response to changes in photoperiod (day-length) and vernalization (long-term exposure to cold temperatures). In the temperate cereals, most of the natural variation in the response to photoperiod is concentrated in the *PPD1* gene (Turner et al. 2005; Beales et al. 2007; Wilhelm et al. 2009) whereas most of the natural variation in the response to vernalization occurs in the *VRN1*, *VRN2* and *VRN3* genes (Beales et al. 2007; Trevaskis et al. 2007; Distelfeld et al. 2009a). Throughout the text, a two letter prefix will be added to gene names to indicate the source species (*Hv, Hordeum vulgare; Ta, Triticum aestivum*; *Tm, T. monococcum*). No prefix will be added when the effect is known to occur in all tested Triticeae species.

VRN1 plays a central role in the regulation of flowering time and is responsible for most of the natural variation in vernalization requirement in wheat and barley. This gene encodes a MADS-box transcription factor with high similarity to the meristem identity genes *APETALA1* (*AP1*), *CAULIFLOWER* (*CAL*) and *FRUITFULL* (*FRU*) (Yan et al. 2003) which promote the transition of the vegetative shoot apical meristem (SAM) to the reproductive phase in Arabidopsis (Ferrandiz et al. 2000). The *TmVRN2* locus includes two similar genes that encode proteins with a putative zinc finger and a CCT domain (*TmZCCT1* and *TmZCCT2*) that acts as flowering repressors, with no clear homologues in Arabidopsis (Yan et al. 2004b). *VRN3* encodes a RAF kinase inhibitor-like protein with high homology to Arabidopsis protein *FLOWERING LOCUS T* (*FT*) (Yan et al. 2006) that acts as a long-distance flowering signal (florigen).

The induction of the SAM to a reproductive stage is prevented in winter wheat and barley varieties by the presence of functional *ZCCT1* or *ZCCT2* genes (reviewed in Trevaskis et al. 2007; Distelfeld et al. 2009a) and a repressed state of the *VRN1* chromatin associated with high levels of histone 3 lysine 27 trimethylation (H3K27me3) (Oliver et al. 2009). Vernalization results in a stable reduction of H3K27me3 and in parallel increases of other histone marks associated with an active chromatin state. The gradual increase in *VRN1* transcripts is associated with the repression of *VRN2* (Loukoianov et al. 2005; Trevaskis et al. 2006) and the up-regulation of *FT* (Distelfeld et al. 2009a). The up-regulation of *FT* requires long days, a process mediated by photoperiod genes *PPD1* and *CO* (Beales et al. 2007; Turck et al. 2008). Long days are also required for the transcription of *ZCCT1* and *ZCCT2* (Dubcovsky et al. 2006; Trevaskis et al. 2006). Null or non-functional alleles for both *ZCCT* genes eliminate the vernalization requirement and result in spring growth habit (Yan et al. 2004b; Distelfeld et al. 2009b).

The strong epistatic interactions observed among the *VRN1*, *VRN2* and *VRN3* (*FT*) genes suggest that they are part of the same regulatory gene network. However, the existence of a feedback regulatory loop including these three genes has complicated the interpretation of different flowering experiments, and resulted in two alternative models for the regulation of flowering in the temperate cereals (Fig. 1). The first model, designated hereafter as the "VRN2-to-FT" model (Fig. 1a), suggests that *VRN2* represses *FT* and that *FT* is a promoter of *VRN1* (Trevaskis et al. 2007; Distelfeld et al. 2009a). The alternative model, designated hereafter as the "FT-to-VRN2" model (Fig. 1b), suggests that *VRN1* promotes *FT* and that *FT* acts as a repressor of *VRN2* (Shimada et al. 2009).

The VRN2-to-FT model (Fig. 1a), suggests that in the temperate cereals, *FT* acts in a similar way as the homeologous genes in Arabidopsis by promoting transcription of the meristem identity genes (Teper-Bamnolker and Samach 2005; Wigge et al. 2005). As in Arabidopsis, the wheat *Ta*FT protein has been shown to interact with an FD-like protein (*Ta*FDL2) that binds to the *TmVRN1* promoter (Li and Dubcovsky 2008). However, this model does not explain well the *TmFT* transcription profiles recently described in the non-flowering *mvp* mutants (Shimada et al. 2009).

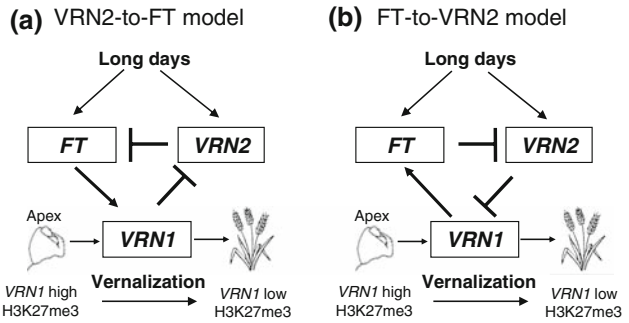

Fig. 1 General model of flowering in wheat. **a** In the model proposed by Distelfeld et al. (2009a, b) and Trevaskis et al. (2007), VRN2 is a repressor of *FT*, which promotes *VRN1* under long days. *VRN1* is up-regulated by vernalization and is a repressor of *VRN2*. **b** In the model proposed by Shimada et al. (2009), *VRN1* is a promoter of *FT* which operates as a repressor of *VRN2*. In this model *VRN2* represses *VRN1*

These non-flowering mutants carry induced deletions that encompass the complete *TmVRN1* coding region and natural deletions of the *TmVRN2* locus (Shitsukawa et al. 2007; Shimada et al. 2009). Recently, Shimada et al. (2009) showed that the *mvp* mutants have very low transcript levels of *TmFT* in spite of the absence of the *TmVRN2* repressor, an unexpected result based on the VRN2-to-FT model (Fig. 1a). To explain this result Shimada et al. (2009) proposed the alternative FT-to-VRN2 model (Fig. 1b), in which *VRN1* promotes *FT* transcription and its deletion explains the down-regulation of *FT*. However, this model contradicts the known interactions between Arabidopsis *FT* and *VRN1* homologues (*AP1/CAL/FUL*).

In order to clarify these conflicting models, we performed a more detailed molecular and genetic characterization of the *mvp* mutants, and generated populations segregating simultaneously for the *TmVRN1* and *TmVRN2* deletions. An additional objective of the current study was to test the *TmVRN2* response to vernalization in the absence of *TmVRN1*.

Materials and methods

Plant material and experimental design

The winter wheat accession G3116 (*T. monococcum* ssp. *aegilopoides* L.) was used as the source of the functional *TmVRN2* allele. This allele was introgressed into spring

T. monococcum ssp. *monococcum* accession DV92 through six backcrosses which resulted in the winter line DV92:G3116-*Vrn2* (Yan et al. 2004b). DV92:G3116-*Vrn2* was crossed with the M_2-1921 line of KU104-2 and M_2-1326 line of KU104-1 (heterozygous for the *mvp-1* and *mvp-2* mutations, respectively). Hybrids from these two crosses were self-pollinated and two F_2 populations, designates hereafter as *pop-1* and *pop-2*, respectively, were generated. Plants from these segregating populations were grown under long days (LD, 16 h light/8 h dark) and constant temperature (20°C). The F_2 plants were genotyped with *TmVRN1* and *TmVRN2* markers (see next section) and only those carrying at least one functional *TmVRN2* copy were selected for the expression experiment. Among those, 12 non-flowering lines homozygous for the *mvp TmVRN1* deletions and 12 with at least one copy of the wild type (WT) *TmVRN1* gene were selected for the experiment. The first leaf samples for RNA extraction were collected when plants were 4 weeks old (4 W) before starting the different temperature treatments. Half of the lines from each genotypic class were maintained at 20°C and the other half were transferred to a cold chamber (5°C, LD) for 6 weeks (vernalization treatment). After 6 weeks, a second set of leaf samples was collected for RNA extraction from the 10-week-old (10 W) vernalized plants (before returning them to 20°C) as well as from the non-vernalized controls.

Genotyping

TmVRN1

The F_2 plants were genotyped for the presence or absence of *TmVRN1* using primers 5′-AGCCACAAGAACCGGG ACTA-3′ and 5′-CCCAAACTTTGCGGTGTATC-3′ that amplified a 339-bp fragment from the *TmVRN1* promoter region (BAC sequence AY188331). In addition, a codominant cleaved amplified polymorphism (CAP) marker was developed for the *TRANSCRIPTIONAL ADAPTOR 2* (*TmADA2*) gene, which is adjacent to the deletion. This marker was used to differentiate plants homozygous and heterozygous for the presence of the *TmVRN1* gene. *TmADA2* primers 5′-GAAGATGCACTTGGAGAAGG-3′ and 5′-GTCTCTTTGCATTGTACCCA-3′ were used to amplify a 708-bp product from the parental lines DNAs. Digestion of the amplified product with restriction enzyme *Taq*I yielded two fragments of 30 and 678-bp in the *mvp* lines (KU104-2 and KU104-1) and three fragments of 30, 185 and 493-bp in the DV92:G3116-*Vrn2* line.

TmVRN2

Since *TmVRN2* is deleted in both *mvp* mutants, we developed a codominant degenerate CAP marker for the adjacent *SNF2* gene (BAC sequence EF540321). Primers 5′-GTGG TTTGATTGGCATTCCT-3′ and 5′-CAGACAACTGCG TCACCGTA-3′ were used to amplify a 187-bp product from the parental lines DNAs. The underlined G indicates a mutation introduced to generate an *Rsa*I restriction site. Digestion of the amplified product with restriction enzyme *Rsa*I yielded three fragments of 19, 75 and 93-bp in the *mvp* lines (KU104-2 and KU104-1) and two fragments of 75 and 112-bp in DV92:G3116-*Vrn*-2 line.

Primer pairs for the genomic copies of the *T. monococcum AGAMOUS-LIKE GENE 1* (*TmAGLG1*), *CYTOCHROME B5* (*TmCYB5*), *CYSTEINE PROTEINASE* (*TmCYS*), and *PHYTOCHROME-C* (*TmPHYC*) genes were described before (Yan et al. 2003).

Quantitative-PCR analysis

RNA was extracted using Spectrum Plant Total RNA Kit (Sigma-Aldrich, Saint Louis, MO, USA). First strand cDNA was synthesized from 1 μg of total RNA with the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA, USA). Quantitative-PCR was performed on an ABI PRISM 7000 SDS (Applied Biosystems, Foster City, CA, USA) using SYBR® GREEN and the previously published primer sets for the *TmZCCT1* and *TmZCCT2* (Distelfeld et al. 2009b) and *TmFT* (Yan et al. 2006). As an endogenous control, a fragment from the *T. monococcum TRANSLATION ELONGATION FACTOR 1* (*TEF1*) gene was amplified with primers 5′-GCCCTCCTTGCTTTCACTCT-3′ and 5′-AACGCGCCTTTGAGTACTTG-3′ (91-bp, efficiency 99%). The $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) was used to normalize and calibrate transcript values relative to the endogenous control.

In addition, we developed primer sets for the RT-PCR studies of *TmPHYC* (5′-GGAGCAGAGGCAACTTTTTG-3′ and 5′-ATTCCACCGTGTTCATCTCC-3′, 119-bp), *TmCYS* (5′-GATCTTGGTCAGCCCTTCC-3′ and 5′-GACCTCA CCGACGAGGAGT-3′, 234-bp), and *TmAGLG1* (5′-GCT GAAGGCAAGAGTTGAGG-3′ and 5′-CTAGGGCCTG GAAGAAGTGC-3′, 259-bp).

Results

Co-segregation of the *mvp* mutations and the non-flowering phenotype

The two F_2 populations (*pop-1* and *pop-2*) segregating for the *mvp-1* and *mvp-2* mutations showed a ratio of three flowering to one non-flowering plants (*pop-1* 49:10 χ^2 $P = 0.46$, *pop-2* 31:8 χ^2 $P = 0.75$). All the plants that failed to flower were homozygous for the *TmVRN1* deletion, whereas all the flowering plants carried at least one wild

Table 1 The different genotypes and phenotypes of *pop-1* and *pop-2* derived from crosses between heterozygous lines for *mvp-1* and *mvp-2* with einkorn wheat accession DV92:G3116-*Vrn2*

Genotype	*pop1*	*pop2*	Phenotype
Homozygous for *mvp-1* deletion	10	8	Inability of the SAM to initiate reproductive phase
At least one copy of *TmVRN1* and null allele of *TmVRN2*	10	9	Spring growth habit, induced to flower without vernalization
At least one copy of *TmVRN1* and functional *TmVRN2*	39	22	Winter growth habit, induced to flower after 6 weeks vernalization

pop1, DV92:G3116-*Vrn2* × M_2-1921 line of KU104-2
pop2, DV92:G3116-*Vrn2* × M_2-1326 line of KU104-1

type *TmVRN1* allele (Table 1). These results indicated that the non-flowering phenotype described before (Shitsukawa et al. 2007) is controlled by a single locus linked to the *mvp-1* and *mvp-2* deletions.

Among the flowering plants (carrying at least one copy of *TmVRN1*), those homozygous for the *TmVRN2* deletion, flowered early and without vernalization (~70 days, spring growth habit) while those with at least one functional copy of *TmVRN2* were induced to flower only after 6 weeks of vernalization (winter growth habit) (Table 1). This indicates that only *TmVRN2* was segregating in these set of plants, and that both parental lines have the recessive *Tmvrn1* allele for winter growth habit. This was confirmed by sequencing of the *TmVRN1* promoter region of the two *mvp* parental lines, which showed 100% identity to the *TmVRN1* gene from winter *T. monococcum* accession G1777 (Yan et al. 2003).

Molecular characterization of the *mvp* mutations

Shitsukawa et al. (2007) inferred that the deletion in the *mvp-1* mutant was limited to a 19-kb region including only the *TmVRN1* gene. This conclusion was based on two primer sets that successfully amplified PCR products from the 5′ (65,228–66,046) and 3′ (84,799–85,280) regions of the *TmVRN1* gene using *mvp-1* DNA. However, our analyses of these primers using the Triticeae repeat database (TREP-http://wheat.pw.usda.gov/ITMI/Repeats/blastrepeats3.html) showed that the 65,228–66,046 sequence was 100% identical to a CACTA DNA transposon (TREP1225) and the 84,799–85,280 sequence 100% identical to a Gypsy retrotransposon (TREP1240). Therefore, the PCR products obtained with these primers are likely to represent products from multiple repetitive loci and are not appropriate to infer the borders of the *mvp-1* and *mvp-2* deletions.

The ion beams used to produce the *mvp* mutants are known to induce large deletions that may include multiple genes (Hase et al. 1999). To test this possibility, we used specific primer pairs for six genes tightly linked to *TmVRN1* (Yan et al. 2003) and tested their presence in the *mvp-1* and *mvp-2* mutants and parental lines (Fig. 2). The PCR products from genes *TmCYB5* and *TmADA2* were

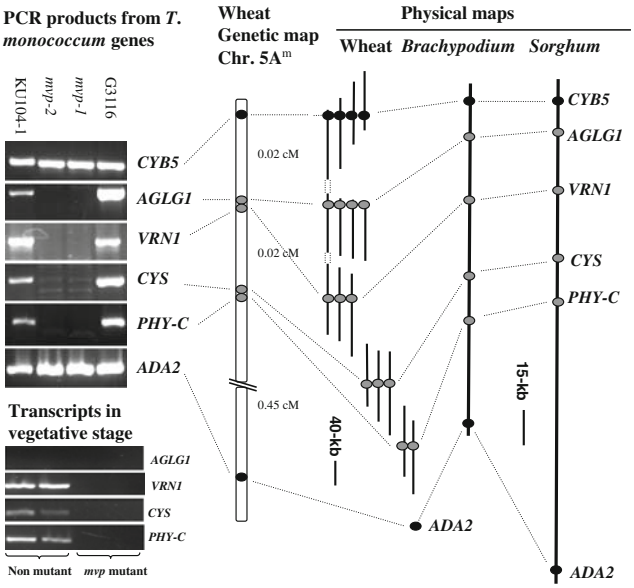

Fig. 2 Molecular characterization of the maintained vegetative phase (*mvp*) deletions. DNAs from diploid wheat accessions G3116, *mvp-1* mutant, *mvp-2* mutant and KU104-1 (WT of the *mvp-1* mutant) were tested for PCR amplification with primers for six genes from the *TmVRN1* physical and genetic maps (Yan et al. 2003). Sequences from the colinear regions in sorghum (205-kb) and *Brachypodium* (140-kb) are shown on the right. The primers for the *TmCYB5* and *TmADA2* genes (*black circles*) successfully amplified products from all DNA samples, whereas primers for *TmAGLG1*, *TmVRN1*, *TmCYS* and *TmPHYC* (*gray circles*) failed to amplify products from the genomic DNAs extracted from the *mvp-1* and *mvp-2* mutants. Transcripts of the *TmPHYC*, *TmCYS*, and *TmVRN1* genes were detected in the leaves of the vernalized plants of non-mutant plants from *pop-1* and *pop-2* but not in those homozygous for the *mvp* deletions. *TmAGLG1* is known to be expressed in young spikes (Yan et al. 2003)

successfully amplified from all samples, but the primers for *TmAGLG1*, *TmVRN1*, *TmCYS* and *TmPHYC*, all failed to amplify any product in the *mvp-1* and *mvp-2* mutants. The same primers amplified fragments of the expected sizes in *T. monococcum* controls G3116 and KU104-1 (Fig. 2). The deleted genes (*TmAGLG1*, *TmVRN1*, *TmCYS* and *TmPHYC*) cover a 0.02 cM region whereas the first genes present outside the deletion (*TmCYB5* and *TmADA2*) are 0.5 cM apart. This indicates that the *mvp-1* and *mvp-2* deletions encompass regions that are 0.02–0.5 cM long.

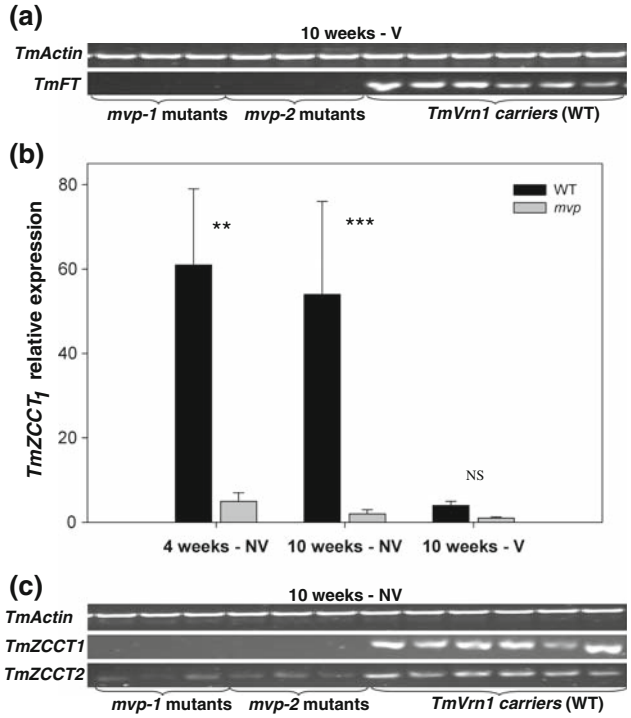

Fig. 3 Transcription profiles associated with the *mvp* mutations. Transcript levels of *TmVRN2* (*TmZCCT1* and *TmZCCT2*) and *TmFT* were compared in leaf samples from F_2 populations derived from a cross between heterozygous maintained vegetative phase mutants (*mvp-1* and *mvp-2*) and diploid winter lines (*TmVRN2* carriers). **a** Semi Q-PCR analysis of plants after vernalization confirmed that the down-regulation of *TmFT* co-segregates with the *mvp* deletions. **b** Expression of *TmVRN2* (*TmZCCT1*) in F_2 plants carrying at least one functional copy of *TmVRN1* (WT, *black bars*) compared with F_2 plants homozygous for the *mvp* deletion (*mvp*, *gray bars*). Comparisons were made at two time points (4 and 10-week-old plants), either with vernalization (*V*) or without vernalization (*NV*). No significant differences were observed between the two populations and therefore the data presented is an average of the two populations. **, *** and NS indicate significance at $P \leq 0.01$, 0.001 or non-significant effect, respectively, using Student's *t* test. **c** Similar down-regulation was observed for the *TmZCCT2* gene in 10-week-old plants without vernalization treatment

Transcription profiles associated with the *mvp* deletions

Shimada et al. (2009) showed that *TmFT* transcripts are significantly reduced in the *mvp-1* mutants relative to sister lines carrying functional *TmVRN1* alleles. This result was extended here to the *mvp-2* mutants and to the segregating lines in *pop1* and *pop2* (Table 1; Fig. 3a), confirming that the down-regulation of *TmFT* is the result of the *mvp* deletion in the *TmVRN1* region and not of mutations in unlinked genes.

The effect of the *TmVRN1* deletion on *TmZCCT1* and *TmZCCT2* transcript levels could not be studied in the original *mvp* mutants because both lines are homozygous for the natural *TmVRN2* deletions (Shimada et al. 2009). The study of the *TmZCCT1* and *TmZCCT2* transcript levels in plants from *pop-1* and *pop-2* carrying these genes and segregating for the *mvp* deletions, yielded a surprising result. The quantitative PCR results showed that *TmZCCT1* and *TmZCCT2* transcript levels were significantly lower ($P < 0.001$) in the *mvp* mutants than in the wild type sister plants even in the absence of vernalization (Fig. 3b). The low *TmVRN2* transcript levels in the *mvp* mutants were similar to those observed in the wild type winter plants after vernalization (Fig. 3b). The differences in *TmZCCT1* and *TmZCCT2* transcript levels were linked to the *mvp* deletions in both populations (Fig. 3c). In summary, the *mvp* deletions were associated with the down-regulation of both *TmVRN2* and *TmFT* genes compared to their wild type sister lines.

We also analyzed the transcript levels of the other genes present in the *mvp* deletions in *pop1* and *pop2* plants with and without the *mvp* deletions. As expected, no expression of the deleted genes *TmAGLG1*, *TmVRN1*, *TmCYS* and *TmPHYC* was detected in the *mvp* mutants (Fig. 2). The F_2 plants with at least one copy without the *mvp* deletions showed abundant transcript levels for *TmCYS* and *TmPHYC* (Fig. 2). *TmVRN1* was expressed at high levels only in the vernalized wild type plants. *TmAGLG1* transcripts were not detected in any of the leaf samples supporting the results from Yan et al. (2003) that concluded that this gene is expressed in the spike after flowering initiation.

Regulation of *TmVRN2* by light

The unexpected down-regulation of *TmFT* and *TmVRN2* observed in the *mvp* mutants parallels the down-regulation of these two genes under short days (Dubcovsky et al. 2006; Yan et al. 2006). In addition, in wheat plants grown under long days, *TaFT* shows higher expression in the light than in the dark (Shimada et al. 2009).

To study the response of *TmVRN2* transcript levels to light, two different experiments were performed. In the first experiment, 8-week-old non-vernalized plants of *T. monococcum* accession G3116 (winter growth habit) previously grown under long day conditions were moved into the dark after 6 h of light (at noon). Analysis of *TmZCCT1* and *TmZCCT2* expression showed a rapid decrease in transcripts level during the 5 h of the experiment (Fig. 4a, b). A mirror image of this result was obtained in the second experiment, in which plants were moved to the light after being kept for 24 h in dark. In this experiment, *TmZCCT1* and *TmZCCT2* transcript levels increased rapidly during the 4 h of exposure to light (Fig. 4c, d).

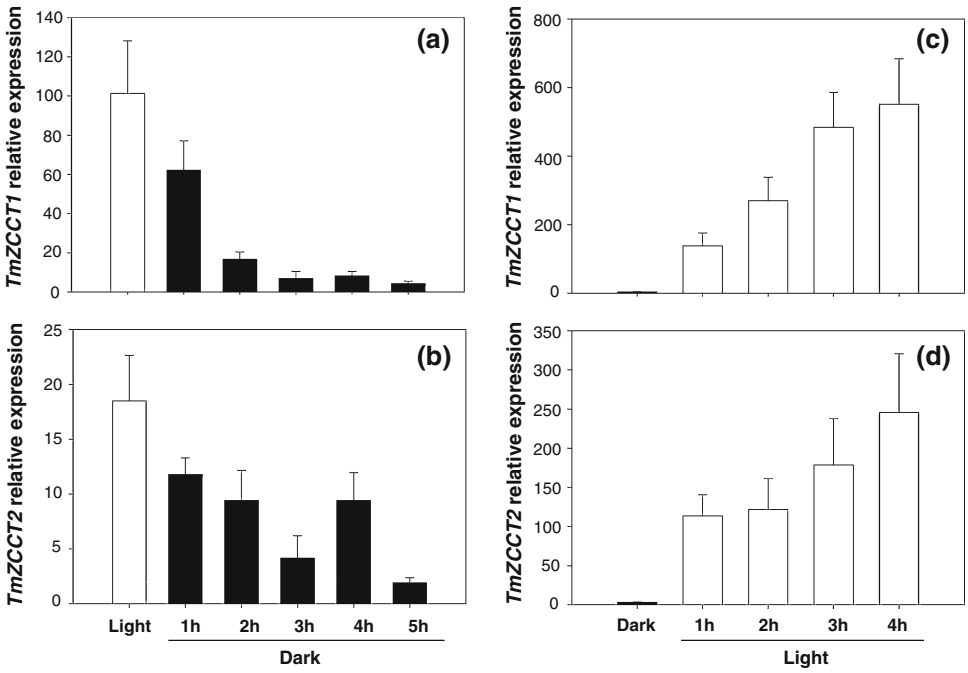

Fig. 4 Expression of *VRN2* (*TmZCCT1* and *TmZCCT2*) under different light conditions in einkorn wheat. Expression of *TmZCCT1* (**a**) and *TmZCCT2* (**b**) when plants were transferred from light to dark. Expression of *TmZCCT1* (**c**) and *TmZCCT2* (**d**) when plants were transferred from dark to normal light

Discussion

Conflicting flowering models

The cloning and characterization of the vernalization genes *VRN1* (Trevaskis et al. 2003; Yan et al. 2003) and *VRN2* (Yan et al. 2004b) was the basis for the first flowering model for temperate cereals. In this initial model *VRN2* was proposed to act as a transcriptional repressor (direct or indirect) of *VRN1*, which is responsible for promoting the transition of the SAM to the reproductive stage. Temperate cereals with a spring growth habit have either deletions or non-functional mutations in the coding region of the *VRN2* repressor (Yan et al. 2004b; Dubcovsky et al. 2005; von Zitzewitz et al. 2005; Distelfeld et al. 2009b) or deletions in regulatory regions of the *VRN1* gene (Yan et al. 2004a; Fu et al. 2005; Hemming et al. 2009). This simple model explains the dominant nature of the *Vrn2* alleles for winter growth habit (one functional copy is sufficient to repress flowering) and the dominant nature of the *Vrn1* mutations for spring growth habit (the deletion of a regulatory region in a single copy results in unregulated promotion of flowering). This model also explains well the known epistatic interactions between these two genes (Takahashi and Yasuda 1971; Tranquilli and Dubcovsky 2000).

Subsequent studies using isogenic lines of hexaploid wheat carrying different combinations of dominant and recessive *TaVRN-A1*, *TaVRN-B1* and *TaVRN-D1* alleles suggested that *VRN1* is able to repress *VRN2* (directly or indirectly) (Loukoianov et al. 2005). In these isogenic lines, the dominant *TaVrn1* alleles are expressed earlier in plant development and are followed later by the down-regulation of *TaVRN2* and the up-regulation of the recessive *Tavrn1* alleles in the other genomes suggesting the existence of a feedback regulatory loop (Loukoianov et al. 2005). The down-regulation of *HvVRN2* after the induction of *HvVRN1* was also observed in barley (Trevaskis et al. 2006).

The discovery that *VRN2* was down-regulated by short days (Dubcovsky et al. 2006; Trevaskis et al. 2006) and that *VRN1* was up-regulated by vernalization under short days both in diploid wheat and barley (Dubcovsky et al. 2006; Trevaskis et al. 2006; Fu et al. 2007), led to the suggestion that *VRN1* was the gene responsible for the perception of the vernalization signal (Trevaskis et al. 2006), which was followed later by other authors (Distelfeld et al. 2009a). Recently, this hypothesis received strong experimental support from the discovery that vernalization is associated with changes in the levels of several histone modifications in the chromatin of *VRN1* but not in the chromatin of *VRN2* or *FT* (Oliver et al. 2009).

The cloning of *VRN3* (Yan et al. 2003) added an additional element to the initial flowering models. This gene is orthologous to rice *Hd3a* and Arabidopsis *FT*, which are both known to encode for proteins (florigen) that travel through the phloem to the SAM and promote flowering (Corbesier et al. 2007; Tamaki et al. 2007). In Arabidopsis, it was demonstrated that the FT protein interacts with FD in the SAM and that this complex binds to the *AP1* promoter (Wigge et al. 2005). A similar mechanism has been postulated in wheat where *Ta*VRN3 (~FT) interacts with *Ta*FDL2 (~FD), which has the ability to bind the wheat *TmVRN1* promoter (Li and Dubcovsky 2008). In the

temperate cereals, *FT* integrates the signals from the photoperiod pathway through its interactions with *PPD1* and *CO* (Turner et al. 2005) and the vernalization pathway through its interactions with *VRN2* (Yan et al. 2006; Hemming et al. 2008; Distelfeld et al. 2009a). Based on the previous results, *FT* was placed in the flowering model as an intermediate step in the *VRN2* repression of *VRN1* (Fig. 1a, VRN2-to-FT model). According to this model the high levels of *VRN2* during the long days of fall prevent the expression of *FT*, which limits the up-regulation of *VRN1*. The up-regulation of *VRN1* during the winter results in the down-regulation of *VRN2*, the release of *FT* from its repression and, under long days, the *FT* up-regulation of *VRN1* transcripts beyond the threshold required to initiate flowering (Fig. 1a).

The model presented in Fig. 1a explains well all the known epistatic interactions among these genes and their expression profiles in different genotypes and environmental conditions. However, this model fails to explain the down-regulation of *TmFT* observed in the *mvp* mutants lacking *TmVRN1* (Shimada et al. 2009). To explain this last result, Shimada et al. (2009) proposed that *TmVRN1* acts as a transcriptional promoter of *TmFT*, and that the absence of *TmVRN1* in the *mvp* mutants is the cause of the down-regulation of *TmFT* (Fig. 1b). Although the FT-to-VRN2 model recognizes the existence of a feedback regulatory loop among the *VRN1*, *VRN2* and *VRN3* genes it reverts the order of the interactions among these genes relative to the VRN2-to-FT model (Fig. 1). Both models can be used to explain most of the experimental results. For example, the up-regulation of *VRN1* transcripts in *FT* over-expressing transgenic plants (Shimada et al. 2009) can be explained by the FT-to-VRN2 model as an indirect effect mediated by the down-regulation of *VRN2*; or by the VRN2-to FT model as a direct up-regulation of *VRN1* by *VRN3* (Fig. 1). The existence of the feedback regulatory loop complicates the interpretation of most of the experiments, since modifications on any of these genes results in direct and indirect effects on the other genes in the loop. The absence of *TmVRN1* in the *mvp* mutants interrupts this feedback loop and, therefore, provides a valuable tool to validate these models.

TmVRN2 expression profiles in the *mvp* mutants conflict with both flowering models

In the FT-to-VRN2 model (Fig. 1b) the deletion of the *VRN1* gene is associated with the down-regulation of *FT*, and therefore it is expected to result in the up-regulation of *VRN2* (this model considers *FT* as a repressor of *VRN2*). To test this prediction, the *TmZCCT1* and *TmZCCT2* transcript levels were studied in the presence and absence of the *TmVRN1* gene.

Surprisingly, in the *pop1* and *pop2* segregating populations the plants homozygous for the *mvp* deletions showed very low *TmZCCT1* and *TmZCCT2* transcript levels, even though the plants were not vernalized and were grown under long days (Fig. 3b). Under the same conditions, the plants carrying functional *TmVRN1* and *TmVRN2* genes, showed *TmZCCT1* transcript levels more than sixfold higher than the plants homozygous for the *mvp* deletions. These plants had winter growth habit and flowered more than 1 month later than the plants homozygous for the *TmVRN2* deletion.

There was a perfect co-segregation between the homozygous *mvp* deletions and the *TmVRN2* down-regulation in the populations segregating for both *mvp* mutations (Fig. 3c) suggesting that this down-regulation is a result of the *mvp* deletions (or a gene linked to it). This result contradicts the expected increase of *VRN2* transcripts in the absence of *VRN1* predicted by both models (Fig. 1a, b) and therefore, it indicates that either both models are incorrect or that the observed changes in *TmVRN2* (and *TmFT*) expression in the lines carrying the *mvp* deletions is the result of other genes deleted in these mutants.

It is interesting to point out here that a simultaneous down-regulation of *VRN2* and *FT* transcript levels is observed when photoperiod sensitive wheat and barley plants are grown under short days (Dubcovsky et al. 2006; Hemming et al. 2008), or when plants grown under long days are in the dark (Shimada et al. 2009). Based on these observations we hypothesized that the gene affecting the *FT* and *VRN2* transcription profile might be associated with light or photoperiod and searched for those genes in the deleted region of the *mvp* mutants.

Genes deleted in the *mvp* mutants of einkorn wheat

In this study, we demonstrated that the deletions present in both the *mvp-1* and *mvp-2* mutants are larger than thought before (Shitsukawa et al. 2007) and include several genes in addition to *TmVRN1* (Fig. 2). Homozygocity for these large deletions co-segregated with the lack of flowering and the down-regulation of *TmVRN2* and *TmFT*, which suggests that these phenotypes are caused by one or several genes present within the *mvp* deletions. Therefore, a description of the genes present within these deletions is relevant to understand the potential candidate genes for these different effects.

The 0.45 cM region between *TmPHYC* and *TmADA2* (Fig. 2) has not been sequenced yet in wheat and therefore, the putative wheat genes present in this region were inferred from the colinear region in *Sorghum bicolor* and *Brachypodium distachyon* (98 and 52-kb, respectively, Fig. 2). This *Sorghum* region has thirteen annotated genes but nine of them are just predicted hypothetical genes and

their predicted proteins show no similarity to any plant proteins (*Sb01g007855/860/874/876/890/910-Sb01g007940*). The other four genes include a member of metal ion binding family (*Sb01g007870*), a gene similar to EMBRYONIC FLOWER 2 (*Sb01g007878*), a gene similar to the isoleucyl tRNA synthetase (*Sb01g007890*) and an oligopeptide transporter (*Sb01g007900*). The *Brachypodium* colinear region includes only six genes, four that are just predicted hypothetical genes, a gene similar to an oligopeptide transporter (*Bradi1g08420*, 58% similar at the protein level to the sorghum orthologue) and a gene similar to the proteinase inhibitor I9 (*Bradi1g08450*). None of these genes seem to be related to the photoperiod or light sensing pathways.

The distal part (0.04 cM) of the *mvp* deletion was sequenced in *T. monococcum* (Yan et al. 2003) and three genes, in addition to *TmVRN1*, were identified; *TmAGLG1*, *TmCYS* and *TmPHYC*. *TmAGLG1* is a MADS-box gene related to the Arabidopsis *AGL2* subgroup and is expressed only in developing spikes (Yan et al. 2003) suggesting that it is an unlikely candidate to regulate flower initiation in the SAM. The *CYSTEINE PROTEINASE* gene (*TmCYS*) belongs to a large family of proteins that are involved with a variety of proteolytic functions in higher plants, particularly those associated with the processing and degradation of seed storage proteins and fruit ripening. They are also induced in response to stresses such as wounding, cold, drought and in programmed cell death and senescence processes (Prins et al. 2008). *TmCYS* transcript levels were abundant in the leaves of the non-*mvp* mutants in our two segregating populations (Fig. 2).

Finally, the *TmPHYC* gene which is also deleted in the *mvp* mutants, belongs to a family of red/far-red photoreceptors that also includes the *PHYA* and *PHYB* genes (Furuya 1993; Devos et al. 2005). Rice *PHYC* mutants exhibit a partial loss of sensitivity to continuous red light and a moderate early flowering under long-day photoperiod (Takano et al. 2005). The *phyC phyA* double mutant shows a dramatic early flowering in rice, suggesting that these genes are implicated in the regulation of flowering repression (Takano et al. 2005). In Arabidopsis, the *PHYC* gene also contributes to variation in flowering time (Balasubramanian et al. 2006), and the *PHYA* and *PHYB* are known to be involved in the posttranscriptional regulation of CO and therefore, contribute to the regulation of the photoperiod pathway (Valverde et al. 2004). The clear association of the *PHYC* gene with the regulation of flowering initiation and the known role of the phytochromes in general on light signaling suggests that the deletion of *TmPHYC* in the *mvp* mutants may have an effects on the regulation of *TmVRN2* and/or *TmFT*. We confirmed here that the *TmPHYC* transcripts are abundant in the leaves of *T. monococcum* (Fig. 2) where *TmVRN2* and *TmFT* are also expressed.

Since several genes are deleted in both *mvp* mutants it is currently not possible to determine which of them is responsible for the lack of flowering and the transcriptional down-regulation of the *VRN2* and *FT* genes. It is possible that different genes within this deletion are responsible for different combinations of the observed phenotypes. Our preferred hypothesis is that the *VRN1* deletion is responsible for the non-flowering phenotype whereas the *PHYC* deletion is responsible for the down-regulation of *VRN2* and *FT* in non-vernalized plants grown under long day conditions.

The putative role of the meristem identity gene *VRN1* on the non-flowering phenotype is supported by a similar phenotype in Arabidopsis triple mutant *ap1 cau ful*, which lacks the three *VRN1* homologues simultaneously (Ferrandiz et al. 2000). However, the *mvp* phenotype seems to be more severe than the Arabidopsis triple mutant, in which limited flowering was observed under heat stress conditions (Ferrandiz et al. 2000). We currently do not know if this stronger flowering repression is the result of the combination of the *TmVRN1* and *TmPHYC* deletions in the *mvp* mutants or simply a stronger effect of the *TmVRN1* deletion in flowering initiation in *T. monococcum*.

The putative role of the *PHYC* gene on the down-regulation of the *VRN2* and *FT* is supported by the rapid response of these genes to light signals. When light conditions change from dark to light or from light to dark, a rapid change in *VRN2* (Fig. 4) or *FT* (Shimada et al. 2009) transcript levels is observed. In addition, all current flowering models propose an effect of photoperiod on the regulation of *VRN2* and *FT* (Fig. 1) (Trevaskis et al. 2007; Distelfeld et al. 2009a). Therefore, if *PHYC* is part of this photoperiodic regulation, no additional changes would be required in the current flowering models to explain the down-regulation of *VRN2* and *FT* in the *mvp* mutants. In contrast, if the down-regulation of *FT* and *VRN2* were the result of the deletion of *VRN1,* then both flowering models (Fig. 1a, b) would require substantial modifications because both predict an up-regulation of *VRN2* in the absence of *VRN1*.

To provide a more conclusive answer to these questions and to determine whether single gene effects can recapitulate the individual *mvp* phenotypes, we have initiated the production of wheat mutants for the *VRN1* and *PHYC* genes using our wheat TILLING populations (Uauy et al. 2009). Until the specific gene(s) responsible for the down-regulation of *VRN2* and *FT* and the non-flowering phenotype are precisely identified, it is premature to use these results to postulate alternative flowering models.

Acknowledgments We would like to thank Dr. Koji Morai for kindly providing us with the heterozygous lines for the *mvp-1* and *mvp-2* mutations. This project was supported by the National Research Initiative grants number 2007-35301-17737 and 2007-35301-18188 from the USDA National Institute of Food and Agriculture. Dr. Assaf

Distelfeld was supported by a Vaadia-BARD Postdoctoral Fellowship Award No. FI-386-06. The authors thank Cindy Miguita and Mei-Yee Lau for excellent technical assistance.

References

Balasubramanian S, Sureshkumar S, Agrawal M, Michael TP, Wessinger C, Maloof JN, Clark R, Warthmann N, Chory J, Weigel D (2006) The *PHYTOCHROME C* photoreceptor gene mediates natural variation in flowering and growth responses of *Arabidopsis thaliana*. Nat Genet 38:711–715

Beales J, Turner A, Griffiths S, Snape JW, Laurie DA (2007) A *Pseudo-Response Regulator* is misexpressed in the photoperiod insensitive *Ppd-D1a* mutant of wheat (*Triticum aestivum* L.). Theor Appl Genet 115:721–733

Corbesier L, Vincent C, Jang SH, Fornara F, Fan QZ, Searle I, Giakountis A, Farrona S, Gissot L, Turnbull C, Coupland G (2007) FT protein movement contributes to long-distance signaling in floral induction of Arabidopsis. Science 316:1030–1033

Devos KM, Beales J, Ogihara Y, Doust AN (2005) Comparative sequence analysis of the *Phytochrome C* gene and its upstream region in allohexaploid wheat reveals new data on the evolution of its three constituent genomes. Plant Mol Biol 58:625–641

Distelfeld A, Li C, Dubcovsky J (2009a) Regulation of flowering in temperate cereals. Curr Opin Plant Biol 12:178–184

Distelfeld A, Tranquilli G, Li C, Yan L, Dubcovsky J (2009b) Genetic and molecular characterization of the *VRN2* loci in tetraploid wheat. Plant Physiol 149:245–257

Dubcovsky J, Chen C, Yan L (2005) Molecular characterization of the allelic variation at the *VRN-H2* vernalization locus in barley. Mol Breed 15:395–407

Dubcovsky J, Loukoianov A, Fu D, Valarik M, Sanchez A, Yan L (2006) Effect of photoperiod on the regulation of wheat vernalization genes *VRN1* and *VRN2*. Plant Mol Biol 60:469–480

Ferrandiz C, Gu Q, Martienssen R, Yanofsky MF (2000) Redundant regulation of meristem identity and plant architecture by *FRUITFULL*, *APETALA1* and *CAULIFLOWER*. Development 127:725–734

Fu D, Szűcs P, Yan L, Helguera M, Skinner J, Hayes P, Dubcovsky J (2005) Large deletions in the first intron of the *VRN-1* vernalization gene are associated with spring growth habit in barley and polyploid wheat. Mol Genet Genomics 273:54–65

Fu D, Uauy C, Blechl A, Dubcovsky J (2007) RNA interference for wheat functional gene analysis. Transgenic Res 16:689–701

Furuya M (1993) Phytochromes—their molecular species, gene families, and functions. Annu Rev Plant Physiol Plant Mol Biol 44:617–645

Hase Y, Shimono K, Inoue M, Tanaka A, Watanabe H (1999) Biological effects of ion beams in *Nicotiana tabacum* L. Radiat Environ Biophys 38:111–115

Hemming MN, Peacock WJ, Dennis ES, Trevaskis B (2008) Low-temperature and daylength cues are integrated to regulate *FLOWERING LOCUS T* in barley. Plant Physiol 147:355–366

Hemming MN, Fieg S, Peacock WJ, Dennis ES, Trevaskis B (2009) Regions associated with repression of the barley (*Hordeum vulgare*) *VERNALIZATION1* gene are not required for cold induction. Mol Genet Genomics 282:107–117

Li C, Dubcovsky J (2008) Wheat FT protein regulates *VRN1* transcription through interactions with FDL2. Plant J 55:543–554

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 25:402–408

Loukoianov A, Yan L, Blechl A, Sanchez A, Dubcovsky J (2005) Regulation of *VRN-1* vernalization genes in normal and transgenic polyploid wheat. Plant Physiol 138:2364–2373

Oliver SN, Finnegan EJ, Dennis ES, Peacock WJ, Trevaskis B (2009) Vernalization-induced flowering in cereals is associated with changes in histone methylation at the *VERNALIZATION1* gene. Proc Natl Acad Sci USA 106:8386–8391

Prins A, Van Heerden PDR, Olmos E, Kunert KJ, Foyer CH (2008) Cysteine proteinases regulate chloroplast protein content and composition in tobacco leaves: a model for dynamic interactions with ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) vesicular bodies. J Exp Bot 59:1935–1950

Shimada S, Ogawa T, Kitagawa S, Suzuki T, Ikari C, Shitsukawa N, Abe T, Kawahigashi H, Kikuchi R, Handa H, Murai K (2009) A genetic network of flowering time genes in wheat leaves, in which an *APETALA1*/*FRUITFULL*-like gene, *VRN1*, is upstream of *FLOWERING LOCUS T*. Plant J 58:668–681

Shitsukawa N, Ikari C, Shimada S, Kitagawa S, Sakamoto K, Saito H, Ryuto H, Fukunishi N, Abe T, Takumi S, Nasuda S, Murai K (2007) The einkorn wheat (*Triticum monococcum*) mutant, *maintained vegetative phase*, is caused by a deletion in the *VRN1* gene. Genes Genet Syst 82:167–170

Takahashi R, Yasuda S (1971) Genetics of earliness and growth habit in barley. In: Nilan RA (ed) Proceedings of the 2nd international barley genetics symposium. Washington State University Press, Washington, pp 388–408

Takano M, Inagaki N, Xie XZ, Yuzurihara N, Hihara F, Ishizuka T, Yano M, Nishimura M, Miyao A, Hirochika H, Shinomura T (2005) Distinct and cooperative functions of phytochromes A, B, and C in the control of deetiolation and flowering in rice. Plant Cell 17:3311–3325

Tamaki S, Matsuo S, Wong HL, Yokoi S, Shimamoto K (2007) Hd3a protein is a mobile flowering signal in rice. Science 316:1033–1036

Teper-Bamnolker P, Samach A (2005) The flowering integrator *FT* regulates *SEPALLATA3* and *FRUITFULL* accumulation in Arabidopsis leaves. Plant Cell 17:2661–2675

Tranquilli G, Dubcovsky J (2000) Epistatic interactions between vernalization genes *Vrn-A^{m}1* and *Vrn-A^{m}2* in diploid wheat. J Hered 91:304–306

Trevaskis B, Bagnall DJ, Ellis MH, Peacock WJ, Dennis ES (2003) MADS box genes control vernalization-induced flowering in cereals. Proc Natl Acad Sci USA 100:13099–13104

Trevaskis B, Hemming MN, Peacock WJ, Dennis ES (2006) *HvVRN2* responds to daylength, whereas *HvVRN1* is regulated by vernalization and developmental status. Plant Physiol 140:1397–1405

Trevaskis B, Hemming MN, Dennis ES, Peacock WJ (2007) The molecular basis of vernalization-induced flowering in cereals. Trends Plant Sci 12:352–357

Turck F, Fornara F, Coupland G (2008) Regulation and identity of florigen: *FLOWERING LOCUS T* moves center stage. Annu Rev Plant Biol 59:573–594

Turner A, Beales J, Faure S, Dunford RP, Laurie DA (2005) The pseudo-response regulator *Ppd-H1* provides adaptation to photoperiod in barley. Science 310:1031–1034

Uauy C, Paraiso F, Colasuonno P, Tran RK, Tsai H, Berardi S, Comai L, Dubcovsky J (2009) A modified TILLING approach to detect induced mutations in tetraploid and hexaploid wheat. BMC Plant Biol 9:115–129

Valverde F, Mouradov A, Soppe W, Ravenscroft D, Samach A, Coupland G (2004) Photoreceptor regulation of CONSTANS protein in photoperiodic flowering. Science 303:1003–1006

von Zitzewitz J, Szűcs P, Dubcovsky J, Yan L, Francia E, Pecchioni N, Casas A, Chen THH, Hayes PM, Skinner JS (2005) Molecular and structural characterization of barley vernalization genes. Plant Mol Biol 59:449–467

Wigge PA, Kim MC, Jaeger KE, Busch W, Schmid M, Lohmann JU, Weigel D (2005) Integration of spatial and temporal information during floral induction in Arabidopsis. Science 309:1056–1059

Wilhelm EP, Turner AS, Laurie DA (2009) Photoperiod insensitive *Ppd-A1a* mutations in tetraploid wheat (*Triticum durum* Desf.). Theor Appl Genet 118:285–294

Yan L, Loukoianov A, Tranquilli G, Helguera M, Fahima T, Dubcovsky J (2003) Positional cloning of wheat vernalization gene *VRN1*. Proc Natl Acad Sci USA 100:6263–6268

Yan L, Helguera M, Kato K, Fukuyama S, Sherman J, Dubcovsky J (2004a) Allelic variation at the *VRN-1* promoter region in polyploid wheat. Theor Appl Genet 109:1677–1686

Yan L, Loukoianov A, Blechl A, Tranquilli G, Ramakrishna W, SanMiguel P, Bennetzen JL, Echenique V, Dubcovsky J (2004b) The wheat *VRN2* gene is a flowering repressor down-regulated by vernalization. Science 303:1640–1644

Yan L, Fu D, Li C, Blechl A, Tranquilli G, Bonafede M, Sanchez A, Valarik M, Dubcovsky J (2006) The wheat and barley vernalization gene *VRN3* is an orthologue of *FT*. Proc Natl Acad Sci USA 103:19581–19586